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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003903132 for a patent by MOLECULAR PLANT BREEDING NOMINEES LTD as filed on 20 June 2003.

WITNESS my hand this
Thirtieth day of June 2004

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SUPPORT AND SALES

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: **Plant promoter**

The invention is described in the following statement:

PLANT PROMOTER

The present invention relates to nucleic acid fragments comprising a plant gene promoter which confers strong pollen-specific expression, and the use thereof for the modification of gene expression in pollen.

The regulation of pollen-specific gene expression has many uses in plant breeding and development. The potential of biotechnology in the development of improved plant cultivars has been recognised in recent years. However, the possibility for transgene escape to wild and non-transformed species raises commercial and ecological concerns. Accordingly, it is desirable to develop mechanisms whereby transgenic plants are male infertile, reducing the potential of cross-pollination with other, non-transgenic species. The regulation of male fertility in plants also has other applications, for example in the maintenance of uniformity of F1 hybrid plants by ensuring that self pollination is minimised during seed production.

Regulation of pollen gene expression also has implications for the production of low allergen plants with respect to pollen allergy. Pollen allergy, in particular grass pollen allergy, is a major environmental disease that afflicts about 20% of the population in cool temperate climates. Millions of Australians suffer from hayfever, with a 62% incidence between ages 15 to 45.

Plant cultivars which have been targeted for improvement using biotechnological methods include forage grasses. Forages are the backbone of sustainable agriculture and contribute extensively to the world economy. Two related genera, *Festuca L.* (fescues) and *Lolium L.* (ryegrasses) are of significant value in temperate grasslands. These genera contain well-adapted, very productive grasses widely distributed in temperate and cool climates in North and South America, Europe, Asia, Australia and New Zealand, where they are used for agricultural and recreational purposes (Jauhar 1993). The commercially most important ryegrasses in cool temperate climates throughout the world are Italian or annual ryegrasses. In New Zealand and Australia, perennial ryegrass is grown on more than 10 million ha providing high quality forage to support over 60 million

sheep and cattle (Siegel et al. 1985). Ryegrass is also responsible for a major portion of grass pollen allergies worldwide.

Accordingly, there is a need for a means for the generation of useful agronomic plants that are male sterile and/or that produce low allergenic pollen.

It is an object of the present invention to overcome, or at least alleviate, one or more of these needs in light of the prior art.

In one aspect, the present invention provides substantially purified or isolated nucleic acid fragments comprising a substantially pollen-specific promoter region, and functionally active variants thereof.

The nucleic acid fragments can be obtained from ryegrass (*Lolium*) or fescue (*Festuca*) species. These species may be of any suitable type, including Italian or annual ryegrass, perennial ryegrass, tall fescue, meadow fescue and red fescue. Preferably the species is a ryegrass, more preferably perennial ryegrass (*L. perenne*).

The applicant has isolated a strong pollen specific promoter from *Lolium perenne* which shows useful properties for targeted pollen specific expression. Such promoters are particularly useful in the production of low pollen allergen transgenic plants, for transgene containment and/or for the down-regulation of the expression of genes that are essential in pollen development to produce male sterile or infertile plants.

The term "isolated" means that the material is removed from its original environment (eg. the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid fragment present in a living plant is not isolated, but the same nucleic acid fragment separated from some or all of the coexisting materials in the natural system, is isolated. Such an isolated nucleic acid fragment could be part of a vector and/or such nucleic acid fragments could be part of a composition, and still be isolated in that such a vector or composition is not part of its natural environment.

By "functionally active" in respect of a nucleotide sequence is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of modifying gene expression in a plant. Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the nucleotides are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least approximately 80% identity to the relevant part of the above mentioned sequence, more preferably at least approximately 90% identity, most preferably at least approximately 95% identity.

By "operatively linked" is meant that the nucleic acid is capable of causing expression of a gene in a plant cell. Usually, the nucleic acid is upstream of the gene. Where a terminator is operatively linked, the terminator is capable of terminating the expressed transcript of the gene. Usually, the terminator is downstream of the gene.

By "an effective amount" is meant an amount sufficient to result in an identifiable phenotypic trait in the plant, or a plant, plant seed or other plant part derived therefrom. Such amounts can be readily determined by an appropriately skilled person, taking into account the type of plant, the route of administration and other relevant factors. Such a person will readily be able to determine a suitable amount and method of administration. See, for example, Maniatis et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, the entire disclosure of which is incorporated herein by reference.

Reference herein to a "gene" or "genes" is to be taken in its broadest context and includes a deoxyribonucleic acid (DNA) sequence which is capable of having its expression regulated by the nucleic acid of the present invention. The term "expression" can relate both to the transcription of ribonucleic acid (RNA) from the DNA, as well as the transcription of RNA followed by the translation of that RNA into an amino acid sequence. Accordingly, a gene includes within its scope both a DNA coding for an amino-acid encoding RNA (i.e. mRNA) as well as a DNA encoding a RNA that does not code for an amino acid sequence. Such an

RNA that does not code for an amino acid sequence may include an antisense RNA. A gene may be of a wild-type or altered form. In the case of an altered gene, the sequence may be modified by alterations to the nucleotide sequence.

It will also be understood that the term "comprises" (or its grammatical variants) as used in this specification is equivalent to the term "includes" and should not be taken as excluding the presence of other elements or features.

In a preferred embodiment of this aspect of the invention, the substantially purified or isolated nucleic acid fragment includes the nucleotide shown in Figure 1 hereto and functionally active fragments and variants of that sequence.

In a further aspect of the present invention there is provided a construct including a nucleic acid according to the present invention. The construct may be a vector. In a preferred embodiment of this aspect of the invention, the vector may include a gene, a nucleic acid according to the present invention and a terminator; said gene, nucleic acid and terminator being operatively linked.

The vector may be of any suitable type and may be viral or non-viral. The vector may be an expression vector. Such vectors include chromosomal, non-chromosomal and synthetic nucleic acid sequences, eg. derivatives of plant viruses; bacterial plasmids; derivatives of the Ti plasmid from *Agrobacterium tumefaciens*, derivatives of the Ri plasmid from *Agrobacterium rhizogenes*; phage DNA; yeast artificial chromosomes; bacterial artificial chromosomes; binary bacterial artificial chromosomes; vectors derived from combinations of plasmids and phage DNA. However, any other vector may be used as long as it is replicable, or integrative or viable in the plant cell.

The terminator may be of any suitable type and may be endogenous to the target plant cell or may be exogenous, provided that it is functional in the target plant cell.

In another embodiment, the vector may include more than one gene. The genes within the same vector may have identical or differing sequences.

A variety of terminators which may be employed in the vectors of the present invention are also well known to those skilled in the art. It may be from the original genomic sequence from which the promoter sequence was isolated or a different genomic sequence. Particularly suitable terminators are polyadenylation signals, such as the CaMV 35S polyA and other terminators from the nopaline synthase (*nos*) and the octopine synthase (*ocs*) genes.

The vector, may include further elements necessary for expression of the nucleic acid, in different combinations, for example vector backbone, origin of replication (ori), multiple cloning sites, spacer sequences, enhancers, introns (such as the maize Ubiquitin Ubi intron), antibiotic resistance genes and other selectable marker genes (such as the neomycin phosphotransferase (*npt2*) gene, the hygromycin phosphotransferase (*hph*) gene, the phosphinothricin acetyltransferase (*bar* or *pat*) gene), and reporter genes (such as beta-glucuronidase (GUS) gene (*gusA*)). The vector may also contain a ribosome binding site for translation initiation. The vector may also include appropriate sequences for amplifying expression.

As an alternative to use of a selectable marker gene to provide a phenotypic trait for selection of transformed host cells, the presence of the vector in transformed cells may be determined by other techniques well known in the art, such as PCR (polymerase chain reaction), Southern blot hybridisation analysis, histochemical GUS assays, northern and Western blot hybridisation analyses.

Those skilled in the art will appreciate that the various components of the vector are operatively linked, so as to result in expression of said gene or genes. Techniques for operatively linking the components of the vector of the present invention are well known to those skilled in the art. Such techniques include the use of linkers, such as synthetic linkers, for example including one or more restriction enzyme sites.

The vectors of the present invention may be incorporated into a variety of plants, including: monocotyledons, such as grasses from the genera *Lolium*, *Festuca*, *Paspalum*, *Pennisetum*, *Panicum* and other forage and turfgrasses, corn,

rice, sugarcane, oat, wheat and barley; dicotyledons, such as arabidopsis, tobacco, soybean, canola, cotton, potato, chickpea, medics, white clover, red clover, subterranean clover, alfalfa, eucalyptus, poplar, hybrid aspen, and gymnosperms (pine tree). In a preferred embodiment, the vectors are used to transform monocotyledons, preferably grass species such as ryegrasses (*Lolium* species) and fescues (*Festuca* species), even more preferably a ryegrass, most preferably perennial ryegrass, including forage- and turf-type cultivars.

Techniques for incorporating the vectors of the present invention into plant cells (for example by transduction, transfection or transformation) are known to those skilled in the art. Such techniques include *Agrobacterium* mediated introduction, electroporation to tissues, cells and protoplasts, protoplast fusion, injection into reproductive organs, injection into immature embryos and high velocity projectile introduction to cells, tissues, calli, immature and mature embryos. The choice of technique will depend largely on the type of plant to be transformed.

Cells incorporating the vectors of the present invention may be selected, as described above, and then cultured in an appropriate medium to regenerate transformed plants, using techniques well known in the art. The culture conditions, such as temperature, pH and the like, will be apparent to the person skilled in the art. The resulting plants may be reproduced, either sexually or asexually, using methods well known in the art, to produce successive generations of transformed plants.

In a further aspect of the present invention there is provided a plant cell, plant, plant seed or other plant part, including, e.g. transformed with, a vector of the present invention.

The plant cell, plant, plant seed or other plant part may be from any suitable species, including monocotyledons, dicotyledons and gymnosperms. In a preferred embodiment the plant cell, plant, plant seed or other plant part is from a monocotyledon, preferably a grass species, more preferably a ryegrass (*Lolium*

species) or fescue (*Festuca* species), even more preferably a ryegrass, most preferably perennial ryegrass, including both forage- and turf-type cultivars.

The present invention also provides a plant, plant seed or other plant part derived from a plant cell of the present invention. The present invention also provides a plant, plant seed or other plant part derived from a plant of the present invention.

In a further aspect of the present invention there is provided a method of modifying gene expression in pollen, said method including introducing into a plant an effective amount of a construct or a vector according to the present invention.

In a preferred embodiment of this aspect of the present invention the nucleic acid according to the present invention is used to direct the pollen-specific expression of a gene to down-regulate the expression of pollen allergens in the plant using antisense technology or other gene silencing technologies. Preferably, the allergen is selected from the major pollen allergens Lol p 1 and Lol p 2. The sequence of Lol p 1 and/or Lol p 2 may be in either a sense or antisense orientation when operably linked with the nucleic acid of the present invention.

Using the methods and materials of the present invention, genes may be targeted for expression in pollen, or the expression of pollen-specific genes may be regulated. For example, gene expression may be increased in pollen by placing a copy or copies of the genes to be expressed operably under the control of the nucleic acid according to the present invention. Alternatively, decreased expression of pollen-specific genes may be achieved by placing an antisense nucleic acid or dsRNA or small interfering RNA (siRNA) derived from the gene operably under the control of the promoter according to the present invention. Furthermore, the nucleic acid of the present invention may be used to introduce a gene into a plant for specific

In a further aspect of the present invention there is provided a chimeric gene comprising the nucleic acid of the present invention operably linked to a gene or genes, such as those encoding pollen allergens. Preferably, the pollen

allergens are the major pollen allergens Lol p 1 and/or Lol p 2. The sequence of Lol p 1 and/or Lol p 2 may be in either a sense or antisense orientation when operably linked with the nucleic acid of the present invention. In a preferred embodiment, the chimeric gene is included in a vector which can be used to transform a plant cell.

In a further aspect of the present invention there is provided a plant cell, plant seed or other plant part including, for example transformed with, a chimeric gene according to the present invention.

The plant cell, plant, plant seed or other plant part may be from any suitable species, including monocotyledons, dicotyledons and gymnosperms. In a preferred embodiment the plant cell, plant, plant seed or other plant part is from a monocotyledon, preferably a grass species, more preferably a ryegrass (*Lolium* species) or fescue (*Festuca* species), even more preferably a ryegrass, most preferably perennial ryegrass, including both forage- and turf-type cultivars.

The present invention also provides a plant, plant seed or other plant part derived from a plant cell of the present invention. The present invention also provides a plant, plant seed or other plant part derived from a plant of the present invention.

In a further aspect of the present invention there is provided a low allergy plant including the chimeric gene of the present invention. In a preferred embodiment, the low allergy plant is a ryegrass.

In a further aspect of the present invention there is provided a method of producing a male sterile plant by introducing into the plant the nucleic acid of the present invention in combination with the gene capable of modulating male fertility. In a preferred embodiment, the gene is a gene critical to pollen development. In a further preferred embodiment, the expression of the gene results in cell death at the site of expression. In a further preferred embodiment the gene encodes the bacterial ribonuclease barnase. The use of the nucleic acid according to the

present invention may enable the specific expression of the relevant gene in pollen, reducing any unwanted side-effects of expression in other plant tissues.

In a further aspect of the present invention there is provided a male sterile plant produced according to the methods according to the present invention. Male sterile plants may be used to develop a transgene containment system by reducing pollen fertility. Furthermore, such male sterile plants may be used in hybrid seed production.

In a further aspect of the present invention there is provided a preparation for transforming a plant comprising a nucleic acid according to the present invention. The preparation may contain vectors or other constructs to facilitate administration to and/or transformation of the plant with the nucleic acid.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the Figures:

Figure 1 shows sequence of genomic clone showing putative promoter and coding regions. Italics (and partially bold): 952bp of genomic sequence; doubly underlined: coding sequence; bold italics, dashed underlined: primer D21pr1L; bold italics: primer D21pr1R (partially doubly underlined). Also shown is the SacI restriction site (bold) and several stop codons in the coding region of the gene (bold underlined).

Figure 2 shows chimeric vectors containing the 952 bp genomic region. A GUS fusion vector driven by the 952 bp genomic region pBS-260gn (Hamilton *et al.* 1992) was used as the basis for the construction of the promoter-reporter cassette for plant cell transformation using PEG-mediated transformation techniques. PBS-260gn contains the GUS reporter gene (Jefferson *et al.* 1987) and the nopaline synthase (nos) terminator sequence. B Vector containing Lol p 1

in an antisense orientation driven by the 952 bp genomic region (pLP2-asLolp1). **C** Vector containing Lol p 2 in an antisense orientation driven by the 952 bp genomic region (pLP2-asLolp2).

Figure 3 shows PCR of putative transgenic tobacco plants containing the Lp952GUS construct using GUS specific primers **B**. Southern hybridisation of the PCR positive plants showing the stable integration of *gusA*.

Figure 4 shows GUS histochemical staining of pollen collected from transgenic tobacco plants containing the Lp952GUS construct.

EXAMPLE 1

Cloning of a novel promoter

In one embodiment of the present invention, a promoter sequence was isolated from a ryegrass gene.

A of 3.9 kb genomic sequence was isolated from a Lambda-DASH II (Stratagene) genomic library constructed from four-week-old perennial ryegrass (*Lolium perenne* L.) cv. Barlano after hybridisation screening of the genomic library with a Lol p2 cDNA sequence. Positive plaques from the tertiary screen were amplified and purified phage DNA was isolated. The genomic region was fully sequenced and found to contain 3.3 kb of promoter and 567 bp of gene sequence which has an ORF of 366 bp and encodes a small protein of 122 amino acids (Figure 1).

EXAMPLE 2

Construction of Chimeric Gene Vectors

A PCR product containing 952 bp of promoter region (SEQ ID 1) was produced using standard PCR conditions. The sequence of the primers follow.

D21pr1L AAAAGTGTGCTGGGATGGTG (SEQ ID 2)

D21pr1R CCATCCAACAAATCCAGAATGGCTTCC (SEQ ID 3)

The 952 bp PCR product was purified, subcloned into pGEMTeasy (Promega), and sequenced to check for PCR amplification errors. A construct was made using the above PCR product as a promoter in fusion with the reporter gene β-glucuronidase (GUS) coding sequence (*gusA*) depicted in Figure 2 A.

The 952 bp PCR product was also used to construct vectors containing the pollen allergen encoding sequences Lol p 1 (Figure 2 B) and Lol p 2 (Figure 2 C) in antisense orientation. These vectors were designed to be capable of silencing the corresponding endogenous genes.

EXAMPLE 3

The Generation of Plant Cells Under Stable Conditions

This chimeric GUS fusion vector was transgenically expressed in the heterologous system tobacco in order to assess the potential expression patterns directed by the 952 bp genomic region. Transgenic tobacco plants were generated by PEG mediated direct gene transfer (DGT) of tobacco protoplasts. The putative transgenic tobacco plants were screened by PCR and Southern hybridisation analysis. The PCR screening was undertaken using *gusA* specific primers for the initial identification of transformed plants (Figure 3A). The PCR positive transgenic plants were then analysed by Southern hybridisation to show stable integration of the transgene (Figure 3B). Southern positive plants were transferred to soil and grown under glasshouse conditions until flowering.

EXAMPLE 4

Assay Promoter Activity in Plant Cells Under Stable Conditions

Tissue samples were collected from the Southern positive plants and screened by histochemical GUS assays to assess the expression pattern of the 952 bp *Lolium perenne* promoter. Expression of the *gusA* reporter gene was observed exclusively in the pollen grains of the transgenic tobacco plants

containing the 952 bpGUS fusion (Figure 4). These results indicate that the 952 bp region of *Lolium perenne* genomic sequence confers strong pollen-specific expression to the *gusA* gene coding sequence and is thus a pollen-specific promoter that represents an excellent candidate for applications requiring targeted gene expression to pollen cells such as transgene containment and/or the down-regulation of pollen allergen genes and/or induction of male sterility.

Those skilled in the art will appreciate that the invention described above is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and products referred to or indicated in this specification, individually or collectively, and any and all combinations of two or more of said steps or features.

Molecular Plant Breeding Nominees Ltd
By their Registered Patent Attorneys

Freehills Carter Smith Beadle

20 June 2003

AGTGTAACCTAGGACTCTAGGCCAGATCGTGACATTGGAATAGTGCACGACATTTCCCTG
CATACGTAACGCCTATGGGTATGGAGCTTCATACCGAGACTCCCAAGTATCATACCAGAG
GGGACGTGGCCTCCTCTATTCTAGGGGCGACGCCACCCCTGGCAATAAAAATAGGAACTT
CTACTAGATAAGGGGGAGGGCTCGAAGCAACAAAGAGGCTAAGAAAGGCGAAAATCAAGC
AAGAACACAAACCCAACAAGCCAGAGCTAAACAAGCCTTAGCACCATGGTCTCTCGCACT
CGAAAATAACGAGGCGAGATGACACTCTTTCCATTCCAACATTTCATAGCTTAGTAGCTA
CCCAAGAGGAGGAACAAGCACCAGCCAACTTCCCGCCGGAAGCGGCACCCACTCAGACTC
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CGATGTATTCTCGGTCCCTAAATTGTATATCTCTCGTGTGCATGTGGATGTTACCCAATG
GAATCGTGGTCACAAGCCCACCACCTACATAAGAATATACAACCGGGAACCAAAACCCTG
ACACTAGGCAAATCATTAGGGCCACGCCGACTATCTCATTCCCGCACGCGTCTAGGTTTC
CCGCCCGTTTCTACCCCTTGTGGGTATCCCCCATCATTCTTGTTTGTATTGGTCCAAAAA
ATCAGCAAAGTTTGCTTTGCCGTGTGTATTCATATAACACTCGATAATGACCCCATCGGC
CTTATTTTTTTATTGTTTCCTTCCTGCCTTTTCTTCCCCGCGATCTTTCAGCCCTTGTGT
CCCTATATATACCCATCTCTCGGATACATAATTCACAACCCACCTCCACCATAAAGTACA
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AATAATAACCTTGGGTGACATAGTTGCCAAAACAAGCTTATATTGTGCACATGTGCGTGT
GTCATGGGACTGGAAAGGGTCGCCGGTGTGAACCACTGATGTGTGCTGCCATTTAGGAAG
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GCAAGTGGGCCTAATATGTGTGTAAAAGTGTGCTGGGATGGTGAGGGGGCCAAGAGCTAG
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FIGURE 1

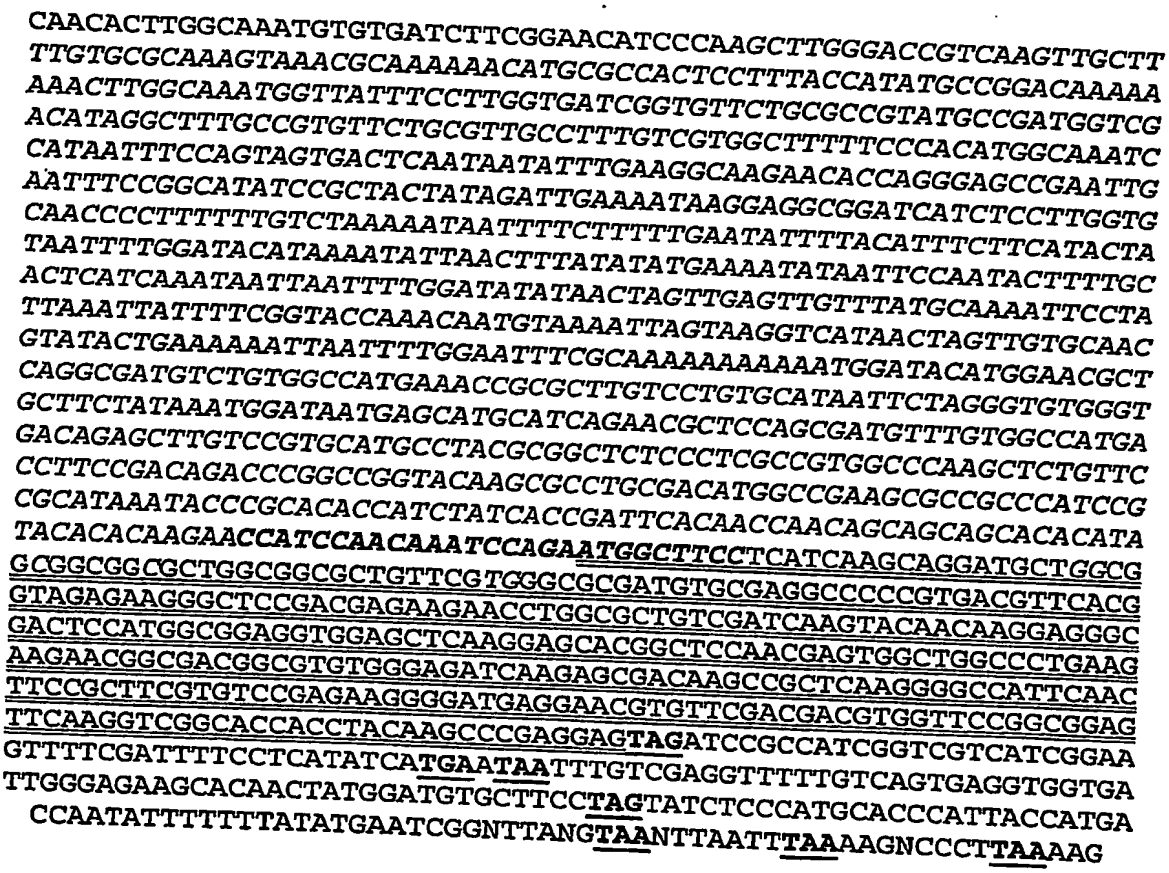
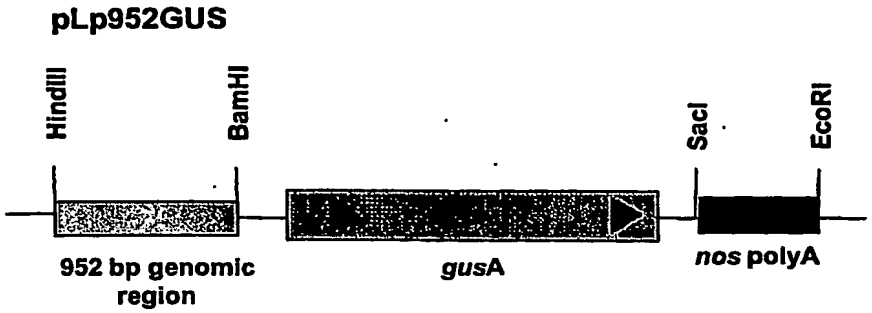
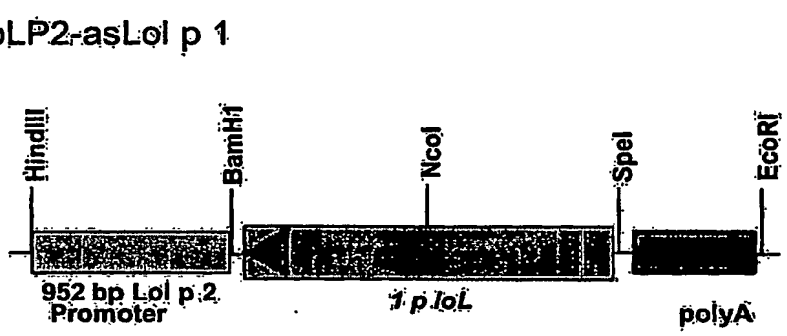


FIGURE 1 (cont.)

A


pLp952GUS
HindIII
BamHI
SacI
EcoRI
952 bp genomic region
gusA
nos polyA

B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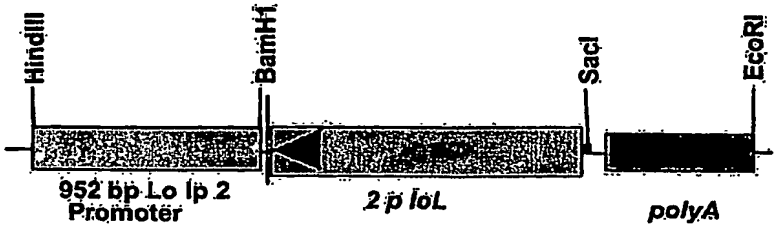
HindIII
BamHI
NcoI
SpeI
EcoRI
952 bp Lol p 2 Promoter
1 p loL
polyA

C


pLP2-asLol p 2
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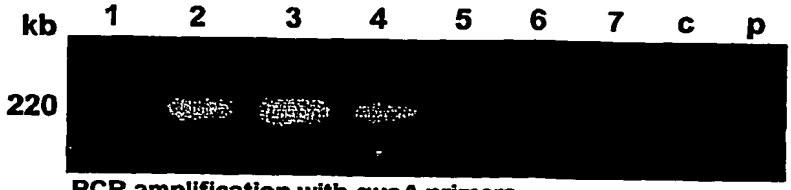
BamHI
SacI
EcoRI
952 bp Lo lp 2 Promoter
2 p loL
polyA

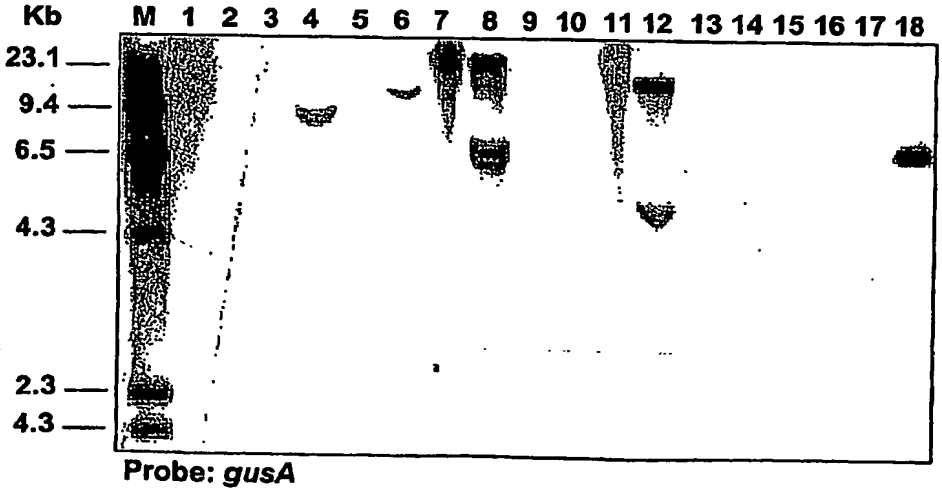
FIGURE 2

A



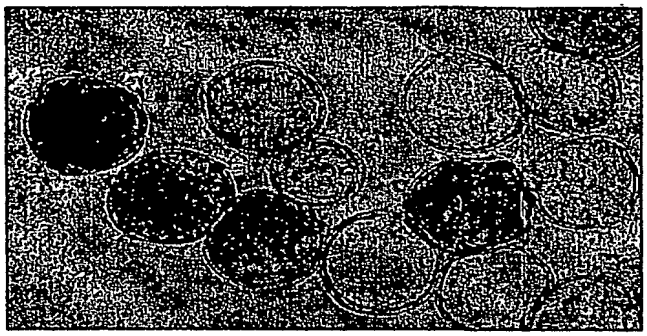
PCR amplification with *gusA* primers

B



Probe: *gusA*

FIGURE 3



Lp952*gusA*

FIGURE 4